

Advances in the molecular regulation of endothelial BMP9 signalling complexes and implications for cardiovascular disease

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Abstract:

Bone morphogenetic protein 9 (BMP9), a member of the transforming growth factor β (TGF β) superfamily is a circulating vascular quiescence and endothelial-protective factor, accounting for the majority of BMP activity in plasma. BMP9 and BMP10 bind preferentially to the high affinity type I receptor activin receptor-like kinase 1 (ALK1) on vascular endothelial cells. Recently, a number of reports have highlighted the important roles of BMP9 in cardiovascular disease, particularly pulmonary arterial hypertension (PAH). *In vivo*, BMP9 activity and specificity are determined by tightly regulated protein-protein recognition with cognate receptors and a co-receptor, and may also be influenced by other proteins present on endothelial cell surface (such as low affinity receptors) and in circulation (such as TGF β family ligands competing for the same receptors). In this review, we summarise recent findings on the role and therapeutic potential of BMP9 in cardiovascular disease, and review the current understanding of how the extracellular protein-protein interaction milieu could play a role in regulating endothelial BMP9 signalling specificity and activity.

Abbreviations:

BMP: Bone morphogenetic protein; TGF β : transforming growth factor β ; ALK1: activin receptor-like kinase 1; PAH: pulmonary arterial hypertension; BMPRII: BMP receptor type II; hPAECs: human pulmonary artery endothelial cells; ENG: endoglin; HHT: hereditary haemorrhagic telangiectasia; AVM: arteriovenous malformation; TAC: transverse aortic constriction; AMD: age-related macular degeneration; ALK1-Fc: ALK1 extracellular domain-Fc fusion protein; GFD: growth factor domain; ActRIIa/b: Activin type II receptor a/b; ActA: activin A; Pro-BMP9: prodomain-bound BMP9; MSCs: mesenchymal stem cells; ECD: extracellular domain; ITC: isothermal titration calorimetry; CV2: crossveinless 2.

Introduction to bone morphogenetic protein (BMP)9 and endothelial BMP signalling

BMP9 is synthesised in the liver(1), circulates in blood at active concentrations, and acts as a serum quiescence factor to maintain vascular homeostasis(2). BMP9 acts selectively on vascular endothelial cells, inhibiting endothelial cell apoptosis, migration, proliferation and angiogenesis(2-4). Dysregulated BMP signalling causes endothelial dysfunction which plays a central role in pulmonary arterial hypertension (PAH), a progressive disease with a poor prognosis. Loss-of-function mutations in BMP receptor type II (*BMPR2*) are the most common genetic cause of PAH(5) and reduced lung vascular expression of BMPRII is a feature of both heritable and idiopathic forms of PAH(6). BMPRII-mediated signalling is pivotal in maintaining endothelial integrity and barrier function. *In vitro*, loss of BMPRII predisposes human pulmonary artery endothelial cells (hPAECs) to apoptosis, increased proliferation and increases permeability of the hPAEC monolayer. *In vivo*, loss of BMPRII causes heightened lung vascular permeability(7).

In humans, there are 15 BMP ligands, among which BMP9 shares high sequence similarity only with BMP10 (64% in the growth factor domain). BMP10, which also signals selectively via the ALK1/BMPRII complex, is exclusively expressed in the right atrium in the adult(8). *Bmp9* *-/-* mice are grossly normal apart from defects in lymphatic vessels and delayed closure of the ductus arteriosus(9-12), whereas *Bmp10* *-/-* mice are embryonic lethal(10). Although there have been no reports of differences between BMP9 and BMP10 signalling in endothelial cells *in vitro*, studies from knockout mice suggest that BMP9 may play a more prominent role in the maintenance of adult vascular homeostasis, whereas BMP10 is indispensable for embryonic development(2, 9-11, 13). The role of BMP10 in adults is still to be elucidated.

Role and therapeutic potential of BMP9 and BMP10 in cardiovascular disease

Compromised function in the BMP9 signalling pathway is linked to a number of cardiovascular diseases. Mutations in the BMP9 receptor *ALK1* and the co-receptor *Endoglin (ENG)* are causal for the human autosomal genetic disorder hereditary haemorrhagic telangiectasia (HHT)(14, 15). HHT is characterised by the presence of multiple arteriovenous malformations (AVM), mostly small telangiectasia on the skin or mucous membranes, but larger AVMs may also be found in lungs, liver and brain. Mutations in *BMP9* itself were also reported in patients with HHT-like vascular anomaly syndrome(16). The pivotal role of endothelial BMP9 signalling in PAH has been strengthened by recent reports which will be discussed further below. In preclinical studies, the beneficial effects of BMP9 signalling have been demonstrated in several other cardiovascular diseases. For example, in a mouse model of left ventricular heart failure induced by transverse aortic constriction (TAC), *Bmp9* knockout mice displayed a more severe fibrotic phenotype, whereas treatment with recombinant BMP9 in wild type mice limited TAC-induced cardiac fibrosis(17). In another report, using adenoviral-mediated gene delivery, BMP9 was shown to prevent oxygen-induced retinal neovascularization in newborn mice and laser injury-induced choroidal neovessel formation in adult mice, suggesting the potential of BMP9 in treating age-related macular degeneration (AMD)(18).

Role of BMP9 in HHT

The important roles of BMP9 and BMP10 in HHT have been reviewed recently(19) and are underpinned by the fact that they are the only known high affinity ligands for ALK1 and ENG (20, 21). *ALK1* and *ENG* mutations found in HHT patients have been tested experimentally, with most resulting in compromised protein folding and secretion(22-25). Those ALK1 or ENG mutants that attain cell surface expression exhibit reduced BMP9 signalling (22, 23). Further evidence comes from a report that mouse pups receiving neutralising antibodies against BMP9 and

BMP10 via lactation developed signs of HHT symptoms, including defects in arteriovenous specification and AVMs (26).

Role of BMP9 in PAH

Genetic evidence strongly supports a pivotal role of compromised endothelial BMP signalling in the development of PAH. Genetic mutations in *BMPR2*, *ALK1* and *ENG* have all been found in patients with idiopathic or heritable forms of PAH(27-30). Recently, a large-scale whole-genome sequencing study in 1038 PAH index cases not only confirmed the mutations in the BMP9 receptors, but also identified heterozygous pathogenic mutations in *BMP9* itself (31). Structural and functional analyses suggested that these mutations lead to reduced cellular secretion of mutant BMP9. Two further studies have confirmed this finding in independent PAH cohorts, with a prevalence of heterozygous *BMP9* mutations as high as 6.7% reported in idiopathic PAH patients from China. In the same report, the authors demonstrated that *BMP9* mutation carriers had low plasma levels of BMP9, supporting loss-of-function as the mechanism for PAH in these patients(31-33). There is a single case report of a homozygous recessive nonsense BMP9 mutation in a case of childhood-onset PAH(34). Interestingly, two PAH patients were identified carrying heterozygous mutations in *BMP10* in a French cohort(32).

There is also strong non-genetic clinical evidence supporting a causal role for reduced BMP9 signalling in PAH. Patients with cirrhosis are at greatly increased risk of developing PAH and cirrhosis patients with advanced liver disease exhibit very low plasma levels of BMP9. The lowest levels are found in cirrhosis patients who develop PAH (known as portopulmonary hypertension)(35). In fact, low plasma BMP9 levels were a good predictor for the presence of PAH in cirrhosis patients. The same investigators showed inhibition of plasma BMP9 and BMP10 with ALK-Fc exacerbated hypoxia-induced pulmonary hypertension in mice(35). These

observations are in keeping with our previous observation, that augmentation of endothelial BMP signalling by recombinant BMP9 reverses established PAH in several rodent models(7). In contrast, Tu *et al.* recently published a study showing that selective BMP9 inhibition partially protects against experimental pulmonary hypertension(36), observations that superficially appear to be at odds with our earlier report(7). Tu *et al.* proposed that BMP9 confers a vasoconstrictive effect by inducing factors such as endothelin-1 and suppressing vasodilation factors such as adrenomedullin and apelin, whereas our report has shown extensively that exogenous BMP9 exerts anti-apoptotic and anti-proliferative effects on vascular endothelial cells, thereby reversing PAH by correcting endothelial dysfunction(7). Nevertheless, the findings by Tu *et al.* are of interest and, if confirmed, provide a starting point to examine whether the chronic loss of BMP9 induces vascular changes typical of HHT - with reduced vascular tone and reduced muscularisation of arterioles, as seen in mice that are heterozygous for a targeted deletion in endoglin gene(37). Such changes would protect rodents from pulmonary vasoconstrictive stimuli, but chronic inhibition of BMP9 might be a hazardous approach in PAH patients, given the strong human genetic evidence that loss-of-function in the BMP9 signalling axis promotes the development of PAH(5).

In summary, human genetics studies support a central role of compromised BMP9 signalling in PAH and HHT, and preclinical studies suggest the beneficial effects of recombinant BMP9 in rodent models of PAH, cardiac fibrosis and AMD. Therefore, a thorough understanding of the regulation of the BMP9 signalling complex at molecular level is essential and will greatly facilitate the bench to bedside development of new therapies targeting this pathway.

Current understanding of BMP9 signalling at a molecular level

Introduction to the components of BMP signalling complexes

BMP9 belongs to the transforming growth factor β (TGF β) superfamily, which is comprised of

over 30 ligands, including BMPs, growth and differentiation factors, activins, and TGF β ligands(38). The TGF β family is highly conserved in eukaryotes, controlling myriad important processes such as embryonic stem cell self-renewal, differentiation, morphogenesis, and tissue homeostasis. Ligands of the TGF β family are generally homodimers, which bind specific transmembrane receptors on the cell surface, inducing the formation of heteromeric complexes consisting two copies of a type I receptor and two copies of a type II receptor. TGF β receptors are serine/threonine kinases, initiating signalling via Smad-dependent and Smad-independent signalling pathways to regulate gene expression(39-41). Tight intracellular and extracellular regulation of TGF β superfamily signalling helps achieve its myriad biological effects.

There are 15 BMPs in humans, signalling via one of four type I receptors (ALK1, ALK2, ALK3 and ALK6) along with one of three type II receptors (BMPRII, ActRIIa and ActRIIb) (Figure 1A). ALK1 is an endothelial-selective type I receptor, mediating the signalling only from BMP9 and BMP10, whilst BMPRII is a type II receptor for most BMPs. ActRIIa/b can mediate signalling from BMPs as well as from other TGF β family ligands, such as activin A (ActA). A high degree of promiscuity has been demonstrated in BMP family ligand-receptor interactions; for example, each ligand can signal through multiple receptor complexes and each receptor pair can mediate the signalling from multiple BMP ligands(42) (Figure 1A). This is largely due to the fact that BMP family ligands and receptors share the same three-dimensional structural fold and signalling complex assembly(40)(and references cited in Table 1). Therefore, different local concentrations of receptors and ligands will have a direct impact on BMP signalling capacity and specificity.

BMPs are synthesised as pre-pro-proteins and processed into prodomains and mature growth factor domains (GFDs) upon secretion(43-45). It has been confirmed for BMP9 and BMP10 that the prodomains remain non-covalently bound to the GFDs in the circulation (4, 46). Due to the lack of commercially available prodomain bound BMPs, current understanding of extracellular BMP-receptor interactions was largely obtained using only BMP GFDs. *In vivo*, prodomain-

bound BMP9 (pro-BMP9) is likely to simultaneously encounter a number of extracellular proteins that might bind itself or its receptors. Figure 1B summarises such extracellular BMP9 protein interaction network. These protein-protein interactions will directly influence BMP9 signalling on endothelial cells.

Structural knowledge of BMP9 signalling

Molecular mechanisms of BMP signalling have been studied at the structural level for many years. Early efforts were focused on the bone-forming BMPs, such as BMP2 and BMP7, which were investigated clinically as a therapy to promote bone reunion following fracture. Although BMP9 was first reported in 2000(1), it did not attract much attention until 2007 when two groups simultaneously reported that BMP9 is a high affinity ligand for ALK1 and preferentially acts on vascular endothelial cells, inhibiting angiogenesis and conferring vascular homeostasis(3, 20). BMP9 is now one of the best studied BMPs, which alongside BMP2, has the most structural data available (Table 1 and Figure 1C), including three crystal structures for BMP9 GFD alone (1ZKZ, 4MPL and 5I05)(24, 47, 48), two crystal structures of the murine prodomain bound to human GFD (4YCG, 4YCI)(44), one crystal structure for the ternary signalling complex of ALK1:BMP9:ActRIIb (4FAO)(25), and one crystal structure for BMP9 bound to the orphan domain of the co-receptor ENG (5HZW)(24). This has greatly facilitated the understanding of BMP9 regulation and signalling at the molecular level. Equipped with such structural knowledge, we can now begin to pinpoint the specific protein-protein interactions that could potentially regulate the BMP9 signalling, and investigate each interaction experimentally in the correct cellular context.

Type I site binding partners and specificity

At physiologically relevant concentrations, BMP9 signalling in endothelial cells is exclusively mediated by the high affinity receptor ALK1(3). At higher BMP9 concentrations and after co-stimulating with lipopolysaccharides or tumour necrosis factor- α , ALK2 can also be activated(49, 50). In non-endothelial cells, ALK2 has been shown to mediate BMP9 signalling by siRNA approaches, such as BMP9-mediated osteogenic signalling activity in mesenchymal stem cells (MSCs) and C2C12 myoblasts, but the nature of the interaction between BMP9 and ALK1 or ALK2 in the context of osteogenic activity is not known(51). In addition, BMP9 signalling through ALK2 has also been shown to promote ovarian cancer cell proliferation(52). The direct binding affinities between BMP9 and seven TGF β family type I receptors have been measured using extracellular domain-Fc fusion fragments and quantitative ELISA(44). Apart from very high affinity for ALK1-Fc, the binding of BMP9 to three other type I receptors in the TGF β family can be detected with three orders of magnitude weaker affinities. Interestingly, although pro-BMP9 showed similar binding affinity for ALK1 to BMP9-GFD, its affinity for ALK2 was too weak to be accurately measured in the same experimental setting(44) (Table 2). It is worth noting that the α 5 helix from the prodomain occupies the type I receptor-binding site in the crystal structure(44)(Figure 1C), which may explain why pro-BMP9 has reduced affinity for ALK2. These data suggest that the prodomain may confer some type I site selectivity, although such selectivity in a cell-based activity assay is yet to be shown.

Type II site binding partners and specificity

The type II site on BMP9 interacts with several partners, including the prodomain, type II receptors and the co-receptor ENG (Figure 1B). Interestingly, these interactions share significant overlap of binding epitopes on BMP9, suggesting they cannot bind BMP9 simultaneously, which has also been confirmed by several Biacore binding studies(21, 44, 53, 54). How the type II receptors displace the prodomain and ENG to form a signalling complex is not known, nor do we

know whether ENG displacement is required for the formation of the signalling complex. The analysis of the reported affinities (Table 2) for these interactions does not prove that such signalling complex formation is solely dependent on the relative affinities. In addition, although the affinities between ALK1-Fc with both BMP9 GFD and pro-BMP9 are of similar high affinity and mostly consistent between reports regardless of the measurement methods, the binding affinities at the type II sites vary significantly between studies even under similar experimental conditions. Moreover, it is important to point out that receptor extracellular domain (ECD)-Fc fusion proteins were used in all studies; Fc fusion proteins are artificial dimers that may not reflect the true binding affinities by receptors on the cell surface. Lastly, all but one of the studies listed have used solid-surface based binding assays, which do not always reflect binding in solution. The only solution study is the binding of BMP9 to its prodomain using isothermal titration calorimetry (ITC) which gave rise to micromolar affinity(44)(Table 2), two orders of magnitude weaker than the affinities measured for prodomain-GFD interactions of BMP5 and BMP10 using Biacore(45). Therefore, more research is required to investigate how specificity is achieved at the type II site and whether any allosteric interaction or conformational change is required for BMP9 signalling complex formation.

Using signalling assays in PAECs, it has been shown that knocking down *BMPR2* alone by siRNA does not lead to a loss of Smad1/5 phosphorylation induced by BMP9 GFD, and simultaneously knocking down both *BMPR2* and *ACTR2a* is required to suppress BMP9-induced Smad1/5 phosphorylation(55). How BMP9 signalling is differentially dependent on BMPRII or ActRIIa remains to be elucidated. In MSCs, it has been shown that expression of ECDs of all three type II BMP receptors, BMPRII, ActRIIa and ActRIIb, can inhibit BMP9-induced osteogenic activity, with ECD of ActRIIb being the most potent. However, MSCs do not express ActRIIb and knocking-down either *ACTR2a* or *BMPR2* in these cells with specific small interfering RNA reduced osteogenic activity of BMP9(56). Because mutations in *BMPR2* but not *ACTR2a/b* are found in

PAH patients, how BMPRII achieves its unique function in endothelial cells that cannot be replaced by ActRIIa/b is of significant medical importance, and how BMP9 achieves its type II site specificity warrants further investigation.

The other protein that binds to the BMP9 GFD type II site is the prodomain (Figure 1B). Although it has been suggested that the prodomain could bind to its cognate BMP with relatively high affinity(45), we have shown that free prodomain does not inhibit BMP9 or BMP10 signalling in endothelial cells(4, 45, 46).

Ligand trap and ligand competition

BMP signalling is also influenced by proteins that function as ligand traps, such as noggin, however, BMP9 and BMP10 are insensitive to noggin inhibition(2, 57). It is unclear whether BMP9 and BMP10 are influenced by other endogenous ligand traps. One candidate is crossveinless 2 (CV2), a chordin family BMP ligand trap(58). It was shown that BMP9/ALK1 signalling in endothelial cells can induce the expression of CV2 and that CV2 can bind BMP9 in pull-down and immunoprecipitation assays. In the same report, the authors provide data showing CV2 inhibits BMP9 induced BRE-luciferase activity in bovine aortic endothelial cells(58). A direct binding between BMP9 and CV2 is yet to be shown using purified proteins.

In addition to ligand traps, other TGF β family members might influence BMP9 signalling in the endothelium. Different TGF β family members can bind to the same type II receptors at the same epitope; this leads to a situation whereby the signalling response to a given TGF β family cytokine is dependent on the expression, affinity, and accessibility of itself as well as all other TGF β family ligands and receptors on specific cells, tissues, and organs. Indeed, Aykul *et al.* revealed that ActA binds ActRIIa/ActRIIb with high affinity and consequently inhibits BMP9 activity in a human liver carcinoma cell line (HepG2) through receptor competition(59). ActA levels are elevated in clinical and experimental pulmonary hypertension(60). Thus, it is possible that ActA, as well as

potentially other circulating TGF β superfamily ligands, could interfere with BMP9 signalling by competing for the type II receptors on endothelial cells. BMP4 and ActA family ligands have both been found in the circulation(60, 61). BMP9 could potentially share BMPRII with BMP4 and ActRIIa/b with ActA family ligands, thus it is essential to establish whether such ligand competition is also present in the context of BMP9 signalling in endothelial cells. Despite BMP9 signalling in HepG2 cells partially inhibited by high concentrations of ActA(59), we found that a 200-fold molar excess of BMP4 or ActA failed to significantly inhibit BMP9 signalling in PAECs. Control experiments showed that ActA is fully active in inducing Smad2/3 phosphorylation in PAECs (Figure 2). The discrepancy between HepG2 and PAECs is probably due to endothelial cells expressing high levels of ALK1 and ENG, which can preferentially capture BMP9 and increase its local concentration at the EC surface. This is another example that the regulation of BMP signalling on target cells is highly context-dependent; we have previously shown that the prodomain of BMP10 can inhibit BMP10 signalling in C2C12 cells but not in PAECs(46). Hence although it is helpful to use model cell lines to dissect receptor and ligand interactions, it is essential to validate the regulation of BMP signalling in the primary, physiologically relevant cell type where cell surface receptor environment also plays a significant role in its signalling capacity and specificity.

Discussion

This review summarised the current advance of BMP9 in cardiovascular disease and reviewed the known extracellular protein-protein interactions that may impact on BMP9 signalling in the endothelium. Many other factors should be considered when evaluating BMP9 interactions with the endothelium. For example: 1) among the target genes induced by BMP9 in endothelial cells, there are a number of intracellular and extracellular BMP signalling modifiers, such as BMP signalling inhibitors *SMAD6*, *SMURF*, ligand trap *NOGGIN* and *BAMBI* etc. Such a feedback loop

seems to be a common theme in TGF β family signalling, emphasizing the importance of maintaining BMP signalling at optimum levels. 2) The organism may regulate BMP9 protein stability and mRNA synthesis; for example, we have shown previously that circulating BMP9 protein itself is subject to redox-dependent proteolysis(48). 3) Given that BMP9 and BMP10 share high affinity receptors on the endothelial surface, BMP10 could potentially compensate for BMP9 to a certain degree(9, 10). However, the prodomains of BMP9 and BMP10 share less than 30% identity, which could potentially affect the distribution of pro-BMP9 and pro-BMP10 on vascular endothelium and possibly also endocardium. 4) It was recently reported the presence of BMP9 and BMP10 heterodimer in plasma, which should signal through the same type I receptor ALK1 in endothelial cells as BMP9 therefore could add another layer of complexity to the regulation of BMP9 signalling(62). 5) Many other cellular signalling pathways are known to crosstalk with BMP9 signalling, such as TGF β , VEGF, Notch, WNT and Hippo signalling pathways(63). The overall physiological outcome of the BMP9 signalling pathway is likely the cumulative effects of different pathways and multiple levels of regulation.

Perspectives

- **The importance of the field**

The circulating BMPs, BMP9 and BMP10, are important players in an emerging axis of vascular endothelial protection. Human genetics studies confirm that loss of signalling via this axis promotes the development of PAH or HHT. Augmentation of circulating BMP9 produced beneficial effects in pre-clinical models of PAH, cardiac fibrosis and AMD, and reversed the loss of functional endothelial BMP9 signalling in rodent models of PAH. A better understanding of the regulation and protein-protein interactions involved in BMP9 signalling complexes will facilitate the therapeutic approaches targeting this pathway in these cardiovascular diseases,

- **Summary of the current thinking**

Human genetic data strongly support a compromised endothelial BMP9/ALK1/BMPRII axis as causative for PAH. In addition, loss-of-function in ENG or ALK1 can cause HHT. Current knowledge from structural biology, protein-protein interactions and signalling assays in human primary endothelial cells have provided a wealth of information on the mechanisms by which BMP9 and BMP10 signal. The specificity and high binding affinity for ALK1 make BMP9 and BMP10 particularly potent ligands on endothelial cells.

- **Future directions**

Future research should aim at understanding the paradox why loss-of-function in the BMP9 signalling axis can on the one hand cause HHT and yet also promote PAH. The type II site selectivity of BMP9 and BMP10 requires further elucidation, not only at the protein-protein interaction level, but also in the endothelial cell signalling context; for example, why mutations in BMPRII but not ActRIIa/b cause PAH. Furthermore, a crystal structure containing BMPRII ECD in complex with a BMP ligand is yet to be reported. Understanding how BMP9 interacts with different type II site binding partners to achieve its endothelial protective function at the molecular level will likely provide new insights into the finely balanced signalling events that are important in endothelial protection.

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Table 1. Reported crystal structures of BMPs and their complexes, shown with Protein Data Bank (PDB) accession codes. In brackets are reference numbers where these structures were reported.

	BMP2	BMP3	BMP6	BMP7	BMP9	BMP11 (GDF11)	BMP14 (GDF5)
Free ligand	3BMP(64) 1REU(65)	2QCQ(66)	2QCW(66) 2R52(67) 2R53(67)	1BMP(68) 1LXI(69)	1ZKZ(47) 4MPL(48) 5I05(24)	5E4G(70) 5UHM(71)	1WAQ(72) 2BHK(73)
Ligand:receptor	1ES7(74) 1REW(65) 2QJ9(75) 2QJA(75) 2QJB(75) 2GOO(76) 2H64(77) 2H62(77)			1LX5(69)	4FAO(25)		3EVS(78) 3QB4(79)
Ligand:co-receptor	4UHY(80) 4UI1(80) 4UI2(80) 4UHZ(80) 4UI0(80)				5HZZ(24)		
Ligand:ligand trap	3BK3(81)			1M4U(82)		5JHW(71)	5HK5(83)
Prodomain-bound complex					4YCG(44) 4YCI(44)		

Table 2. Summary of current affinity measurements between BMP9 and its binding proteins

K _d or EC ₅₀ (nM)*	BMP9 GFD	Pro-BMP9	Notes
ALK1-Fc	0.023±0.0009 <0.008 0.031	0.039±0.01 <0.006	20°C, ELISA binding assay, EC ₅₀ . Mi <i>et al.</i> (44) 37°C, Biacore. Kienast <i>et al.</i> (53) 37°C, Biacore. Townson <i>et al.</i> (25)
ALK2-Fc	70±40	>100	20°C, ELISA binding assay, EC ₅₀ . Mi <i>et al.</i> (44)
BMPRII-Fc	0.04±0.02 3.3 2.1 7.44	1.6±0.7 3.2	20°C, ELISA binding assay, EC ₅₀ . Mi <i>et al.</i> (44) 37°C, Biacore. Kienast <i>et al.</i> (53) 37°C, Biacore. Townson <i>et al.</i> (25) 25°C, Biacore. Aykul & Martinez-Hackert(59)
ActRIIb-Fc	0.04±0.02 1.4 0.033 0.881	0.2±0.1 0.8	20°C, ELISA binding assay, EC ₅₀ . Mi <i>et al.</i> (44) 37°C, Biacore. Kienast <i>et al.</i> (53) 37°C, Biacore. Townson <i>et al.</i> (25) 25°C, Biacore. Aykul & Martinez-Hackert(59)
ActRIIa-Fc	0.5±0.2 42.7 10.37	>100 19.7	20°C, ELISA binding assay, EC ₅₀ . Mi <i>et al.</i> (44) 37°C, Biacore. Kienast <i>et al.</i> (53) 37°C, Biacore. Townson <i>et al.</i> (25)
sENG-Fc	2.7 0.032	1.5	37°C, Biacore. Kienast <i>et al.</i> (53) 20°C, Biacore. Castonguay <i>et al.</i> (21)
sENG**	9.81		ForeBio Octet Red96, Saito <i>et al.</i> (24)
prodomain	800-1000		15°C, ITC. Mi <i>et al.</i> (44)

*Affinities are either expressed as EC₅₀ measured by ELISA-based plate assays, or K_d measured by Biacore, Octet or isothermal calorimetry (ITC).

**sENG: soluble endoglin

Figure legends:

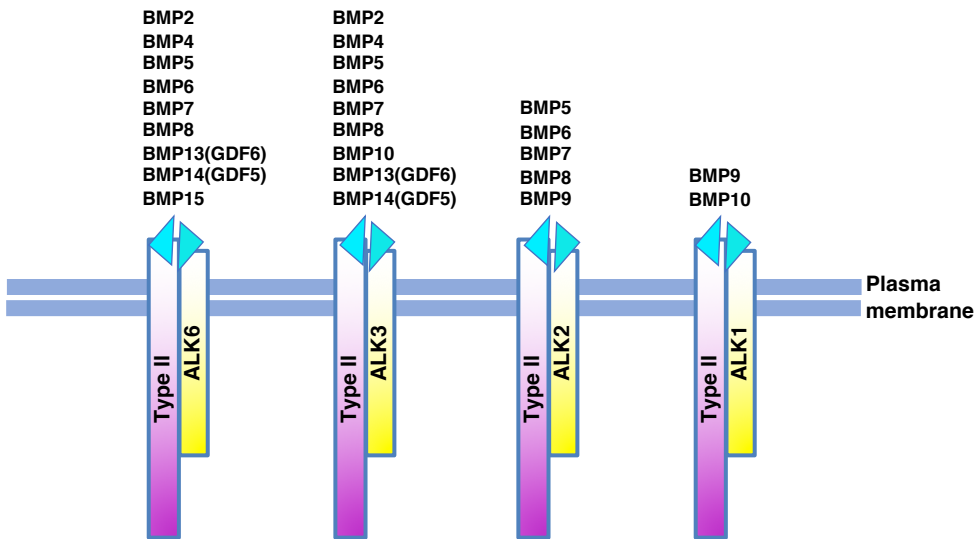
Figure 1 (A) Promiscuity in BMP family ligand-receptor recognition. The same BMP can signal through different receptor pairs, and similarly, the same receptor pair can mediate the signals from different BMPs. Data taken from summary in Yadin *et al*(42). Type II receptors could be BMPRII, ActRIIa or ActRIIb. **(B) Extracellular protein-protein interaction network directly or indirectly involved in BMP9 signal transduction.** BMP9 interacts with ALK1 or ALK2 through residues at the type I site. Type II site on BMP9 can form direct protein-protein interactions with multiple proteins, including BMP9 prodomain (BMP9pro), type II receptors (ActRIIa/b, BMPRII), cell surface and soluble ENG. CV2 has been shown to inhibit BMP9 activity, and crystal structure of CV2:BMP2 demonstrates that CV2 binds BMP at both type I and type II sites (81). BMPRII and ActRIIa/b also bind directly to other TGF β family ligands, therefore ligand competition for the type II receptors could potentially regulate BMP9 activity(59). Cyan: ligands; Yellow: proteins that interact with the type I site on the ligands; Coral: proteins that interact with the type II site on the ligands. Lines indicate direct protein-protein interactions. **(C) Crystal structures of BMP9 and its complexes.** Each protein molecule is coloured in the same way as the label below. Type I and type II receptor binding sites are highlighted.

Figure 2. Activin A and BMP4 do not inhibit BMP9 signalling in hPAECs. (A) Serum-starved hPAECs were pre-treated with PBS or ActA (at 10x, 25x, 50x, 100x and 200x molar excess of BMP9) for 10 minutes to allow ActA pre-binding before treatment with PBS or BMP9 (at 0.3 ng/ml GFD concentration) for an additional 10 minutes, following which the cells were harvested for pSmad1/5 or pSmad2 immunoblotting. Since such a short time treatment will not cause change in total Smad levels, α -tubulin probed on the same blot was used as loading controls. Band intensity of ActA effects on BMP9-induced Smad1/5 phosphorylation from three independent experiment were quantified using Image J and shown on the right. **(B&C)** Serum-

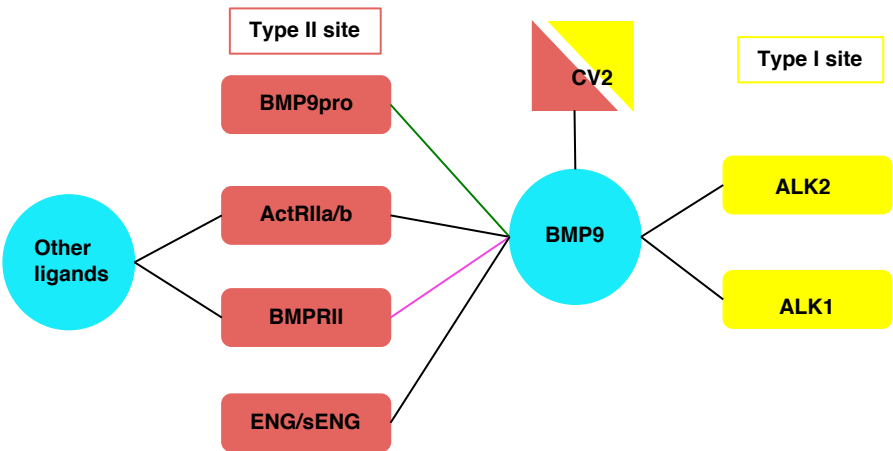
starved hPAECs were pre-treated with PBS or ActA (at 25x, 50x and 100x molar excess of BMP9) for 10 minutes and then treated with PBS or BMP9 (at 0.3 ng/ml GFD concentration) for either an additional 1 hour before qPCR analysis of *ID2* (B) or an additional 5 hours before qPCR analysis for *BMP2* (C) mRNA expression, N=5. **(D)** Serum-starved hPAECs were pre-treated with PBS or BMP4 (at 10x, 25x, 50x, 100x and 200x molar excess of BMP9) for 10 minutes and then treated with PBS or BMP9 (at 0.3 ng/ml GFD concentration) for an additional 10 minutes, following which the cells were harvested for pSmad1/5 immunoblotting. Alpha-tubulin was used as loading control. One representative blot from three experiments is shown, with band intensity of BMP4 effects on BMP9-induced Smad1/5 phosphorylation quantified using Image J and shown on the right, N=3. **(E&F)** Serum-starved hPAECs were pre-treated with PBS or BMP4 (at 25x, 50x and 100x molar excess of BMP9) for 10 minutes and then treated with PBS or BMP9 (at 0.3 ng/ml GFD concentration) for either an additional 1 hour before qPCR analysis of *ID2* (E) mRNA expression or an additional 5 hours before qPCR analysis for *BMP2* (F) mRNA expression, N=5. All graphs are shown as means \pm S.E.M.

Figure 1

A



B



C

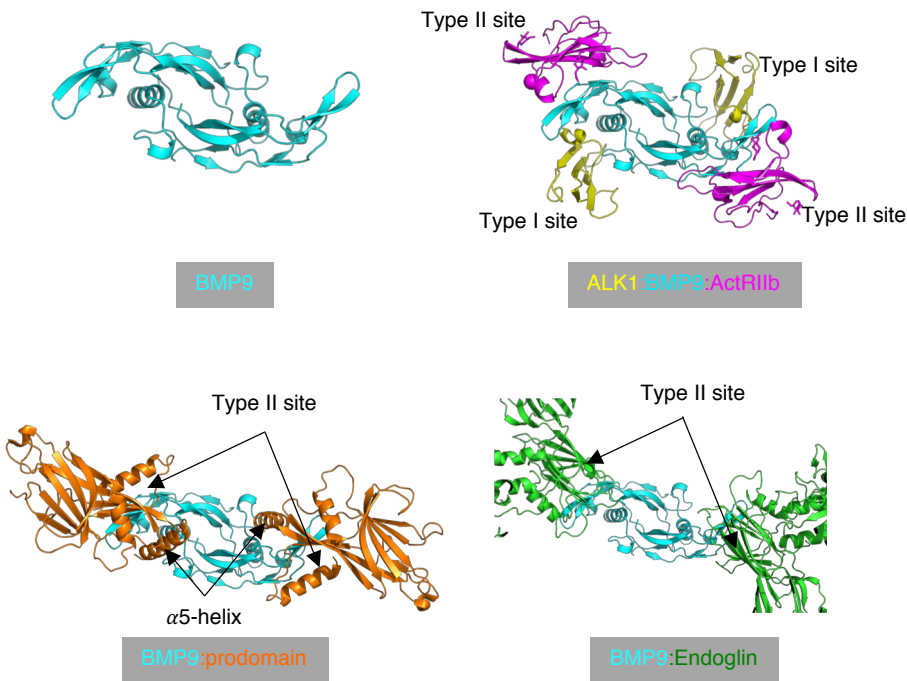


Figure 2

